REMARKS

Claims 28-32 have been cancelled. Claims 5 and 14 have been amended. Claims 5, 9, 12, 14, and 16 are now pending in this application. The amendments have been made to clarify the claimed subject matter and to correct typographical errors. Accordingly, the amendments do not constitute the addition of new matter. Applicant respectfully requests the entry of the amendments and reconsideration of the application in view of the amendments and the following remarks.

Corrections to the specification

The specification at page 18, line 27 has been corrected to insert the SEQ ID NOS. of the primers. The specification at page 20 has been corrected to change "n°" to "No." A typographical error has been corrected.

Formal drawings

Applicant submits formal drawings herewith.

Rejection under 35 U.S.C. § 112, first paragraph

Claims 5, 9, 12, 14, and 16 are rejected under 35 U.S.C. § 112, first paragraph because the specification, while being enabling for two human polynucleotides consisting of SEQ ID NO: 1 and 10, a rat polynucleotide of SEQ ID NO: 3 and a mouse polynucleotide of SEQ ID NO: 5 that encode peroxisomal -associated polypeptides corresponding to SEQ ID NOS: 2, 4, and 6, respectively, and polynucleotide probes of SEQ ID NOS: 7-9, and 11-16 for in vitro diagnosis, does not enable the skilled art worker to make and use the invention for the scope as claimed.

The Examiner has stated that the specification is enabling for human polynucleotides consisting of SEQ ID NO:1. Claim 5 has been amended to recite an isolated or purified polynucleotides ***consisting of*** SEQ ID NO:1 or its complementary strand. The subject matter of claim 5 is clearly enabled by the specification, which discloses a human polynucleotide, having SEQ ID NO:1. Applicants kindly request withdrawal of the claim rejection on this basis.

Claim 9 recites a vector comprising the polynucleotide of claim 5. Claim 14 refers to a pharmaceutical composition comprising the nucleotide sequence of claim 5. Claim 16 refers to a cell transformed by the vector according to claim 9.

. While the Examiner has conceded that SEQ ID NO: 1 is enabled by the specification, the Examiner maintains that claims drawn to a pharmaceutical composition comprising the polynucleotide sequence of SEQ ID NO: 1 are not enabled because the inventors have not demonstrated a dose-dependent neuroprotection against excitotoxic brain lesions by peroxiredoxin 5. This ground of rejection is traversed by Applicants' first example, discussed below, which shows that the systemic administration of recombinant peroxiredoxin 5 to mice induced a dose-dependent neuroprotection against excitotoxic brain lesions.

The Examiner further states that a pharmaceutical composition is unpredictable because (1) the efficacy of gene therapy has not been demonstrated, (2) it is not always possible to extrapolate from in vitro diagnostic experiments to in vivo treatment (3) high level expression of genes may not persist (4) appropriate expression of polynucleotide transfer to specific cell types has not been demonstrated, (5) adverse reactions may occur, and (6) gene transfer with naked DNA has low efficiency.

In response, means to deliver genetic material for therapeutic purposes were known at the time of the claimed invention, as demonstrated by the attached patent document (U.S. Patent No. 6,468,798) provided as Attachment A. The '798 patent describes delivery of genetic material for in vivo gene therapy, particularly delivery to the lung for pulmonary disorders. Thus, means to introduce genetic material into mammals were known. The lengthy listing of cited prior art in the front of the '798 patent also attests to the fact that methods of ex vivo gene transfer were well known. Thus, it was well within the skill level in the art to use the polynucleotide of claim 5 in a pharmaceutical composition for gene delivery.

Furthermore, Applicants' claimed composition does not need to work perfectly in order to meet the requirements of 35 U.S.C. § 112, first paragraph. Thus, the Examiner's concerns that high level expression may not persist or that adverse reactions may occur or that the efficiency may be low are misplaced. Clearly, gene delivery by a variety of means with a measurable level of expression in the target cell was not out of reach at the time of the claimed invention.

Furthermore, PRDX5 in PBS is an example of a pharmaceutical composition, comprising a pharmaceutically acceptable carrier (PBS) and the nucleotide sequence of claim 5, which

encodes a polypeptide (PRDX5). Consequently, claims drawn to a pharmaceutical composition are fully supported and enabled by the present specification.

The following examples, which were also previously submitted in reply to the Office Action of June 18, 2002, are presented. In the first example, the Examiner's concern that a dose-dependent neuroprotection against excitotoxic brain lesions by peroxiredoxin has not been demonstrated is directly addressed.

Peroxiredoxin PRDX 5 (PRDX5) belongs to a family of peroxidases widely distributed in eukaryotes and prokaryotes, the peroxiredoxins. The sequence encoding PRDX5 (Accession no NM_012094) 100% corresponds to SEQ ID NO:1 sequence.

In a first example, the inventors have demonstrated that the systemic administration of recombinant peroxiredoxin 5 to mice induced a dose-dependent neuroprotection against excitotoxic brain lesions. In this example, human PRDX5 cDNA (sequence identical to SEQ ID NO:1) was PCR amplified. The PCR product was digested and further ligated into the pQE-30 expression vector. The resulting vector was used to transform *Escherichia coli* strain M15 (pRep4). Bacteria were grown, pelleted cells were lysed by sonication and clarified by centrifugation. The supernatant containing the recombinant PRDX5 was loaded on a column and eluted. The eluted protein was then dialysed against PBS. Excitotoxic brain lesions were induced by intracerebral injection of ibotenate into developing mouse brains. Recombinant PRDX5 (0.1-20 mg/kg) was administered by intraperitoneal injection. Systemically administered PRDX5 induced a dose-dependent neuroprotection of the excitotoxic brain lesions.

As described above an *E. coli* strain was transformed with a vector comprising the sequence of PRDX5 (identical to SEQ ID NO:1). The above example clearly shows the utility of the presently claimed invention. Applicants assert that the example above demonstrates that one skilled in the art could make and use a vector comprising the polynucleotide of claim 5 as recited in claim 9 and a cell transformed by a vector as recited in claim 16. Consequently, the present specification is fully enabling for these claims.

Furthermore, claim 5 has been amended to recite that the polynucleotide consists of SEQ ID NO: 1. The above example relates to PRDX5 which corresponds 100% to SEQ ID NO: 1. Consequently, the above example is commensurate in scope with the claims as presently amended.

Applicants further assert that the above-described experiment also enables a pharmaceutical composition as recited in claim 14. The PRDX5 in PBS is an example of a pharmaceutical composition, comprising a pharmaceutically acceptable carrier (PBS) and the nucleotide sequence of claim 5, which encodes a polypeptide(PRDX5).

In addition, in reply to the Office Action of June 18, 2002, a paper was submitted entitled "Overexpression of human peroxiredoxin 5 in Chinese Hamster Ovary cells" wherein is demonstrated that overexpression of PRDX5 in host cells (Chinese hamster ovary cells) decreases the damage induced by peroxides. For convenience, this paper is resubmitted here as Attachment B.

In this paper it is described that Chinese hamster ovary cells are stably transfected with a vector comprising the cDNA sequence of PRDX5 (accession NO NM_012094) which is identical to SEQ ID NO:1. This second example also provides enablement for claims 9 and 16 relating to a vector comprising the polynucleotide consisting of SEQ ID NO:1 and a cell transformed by said a vector, respectively.

This paper illustrates that overexpression of PRDX5 by Chinese hamster ovary cells in cytosolic and mitochondrial compartments increases cell survival of cells challenged by peroxides and that overexpression of PRDX5 in the nucleus decreases DNA damages induced by peroxides. Thus, PRDX5, which is encoded by a sequence 100% identical to SEQ ID NO:1 has been demonstrated herein to have important applications in therapies. Consequently, this example provides a written enablement for claim 14, which relates to a pharmaceutical composition comprising a pharmaceutically acceptable carrier and the nucleotide consisting of SEQ ID NO:1 (PRDX5 sequence). Thus, one skilled in the art would know how to use the polynucleotide of claim 5 as a pharmaceutical composition.

Furthermore, claim 5 has been amended to recite that the polynucleotide consists of SEQ ID NO: 1. The above example relates to PRDX5 which corresponds 100% to SEQ ID NO: 1. Consequently, the above example is commensurate in scope with the claims as presently amended.

Furthermore, claim 12, referring to a diagnostic device comprising the polynucleotide of claim 5, is enabled by example 2 provided in the present patent application. Example 2 illustrates the detection of a polypeptide encoded by the nucleotide consisting of SEQ ID NO:1 of the present invention in different human tissues. For detection of this polypeptide, the nucleotide consisting of SEQ ID NO:1, according to certain aspects of the invention or at least certain portions thereof, is used in a hybridization protocol for diagnosing the presence of the polypeptide encoded by the nucleotide consisting of SEQ ID NO:1. In view hereof, it is believed that a diagnostic device comprising the nucleotide consisting of SEQ ID NO:1 is sufficiently enabled in the present application.

In view of Applicants' amendments and arguments, reconsideration and withdrawal of the above ground of rejection is respectfully requested.

Claims 5, 9, 12, 14, 16, and 32 are rejected under 35 U.S.C. § 112, first paragraph as containing subject matter that was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s) has possession of the claimed invention at the time that the application was filed.

This ground of rejection is believed to be overcome by Applicants' cancellation of claim 32, amendment of claims 5 and 14 and arguments presented above. Withdrawal of the above ground of rejection is respectfully requested.

Claims 5, 9, 12, 14, 16, and 32 are rejected under 35 U.S.C. § 112, first paragraph as containing subject matter that was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s) has possession of the claimed invention at the time that the application was filed. This is a new matter rejection.

This ground of rejection is believed to be moot in view of Applicants' amendment to claim 5. Withdrawal of this ground of rejection is respectfully requested.

CONCLUSION

In view of Applicants' amendments to the claims and the foregoing Remarks, it is respectfully submitted that the present application is in condition for allowance. Should the Examiner have any remaining concerns which might prevent the prompt allowance of the application, the Examiner is respectfully invited to contact the undersigned at the telephone number appearing below.

Please charge any additional fees, including any fees for additional extension of time, or credit overpayment to Deposit Account No. 11-1410.

Respectfully submitted,

KNOBBE, MARTENS, OLSON & BEAR, LLP

Dated: June 11, 2003

By: _____

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Annexe 1

ROYAUME DE BELGIQUE

MINISTERE DES AFFAIRES ECONOMIQUES

BREVET D'INVENTION

NUMERO DE PUBLICATION : 1011331A6

NUMERO DE DEPOT : 09700692

Classif. Internat. : C07K

Date de délivrance le : 06 Juillet 1999

Le Ministre des Affaires Economiques,

Vu la loi du 28 Mars 1984 sur les brevets d'invention, notamment l'article 22;

Vu l' arrêté royal du 2 Décembre 1986 relatif à la demande, à la délivrance et au maintien en vigueur des brevets d'invention, notamment l'article 28;

Vu le procès verbal dressé le 20 Août 1997 à 15H20 à l 'Office de la Propriété Industrielle

ARRETE:

ARTICLE 1.- Il est délivré à : UNIVERSITE CATHOLIQUE DE LOUVAIN Halles Universitaires; UNIVERSITE DE MONS-HAINAUT place de l'Université 1, B-1348 LOUVAIN-LA-NEUVE(BELGIQUE); place du Parc 20, B-7000 MONS (BELGIQUE)

représenté(e)(s) par : VAN MALDEREN Joëlle, OFFICE VAN MALDEREN, Place Reine Fabiola 6/1 - B 1083 BRUXELLES.

un brevet d' invention d' une durée de 6 ans, sous réserve du paiement des taxes annuelles, pour : POLYPEPTIDE ASSOCIE AU PEROXYSOME, SEQUENCE NUCLEOTIDIQUE ENCODANT LEDIT POLYPEPTIDE ET LEUR UTILISATION DANS LE DIAGNOSTIC ET/OU LE TRAITEMENT DE MALADIES OU DE LESIONS PULMONAIRES.

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ARTICLE 2.- Ce brevet est délivré sans examen préalable de la brevetabilité de l'invention, sans garantie du mérite de l'invention ou de l'exactitude de la description de celle-ci et aux risques et périls du(des) demandeurs(s).

Bruxelles, le 06 Juillet 1999
PAR DELEGATION SPECIALE :

L. W...
CONSE...

09700692 A6

POLYPEPTIDE ASSOCIE AU PEROXYSOME, SEQUENCE NUCLEOTIDIQUE ENCODANT LEDIT POLYPEPTIDE ET LEUR UTILISATION DANS LE DIAGNOSTIC ET/OU LE TRAITEMENT DE MALADIES OU DE LESIONS PULMONAIRES

Objet de l'invention

La présente invention est relative à un nouveau polypeptide associé au peroxysome, la séquence nucléotidique encodant ledit polypeptide et les fractions de celle-ci ainsi que leur utilisation pour le diagnostic et/ou le traitement de maladies ou de lésions pulmonaires.

Arrière-plan technologique à la base de l'invention

Les peroxysomes, également dénommés "microbodies", sont des organites intracellulaires qui diffèrent des mitochondries et des chloroplastes présents dans les cellules eukaryotes. Ces organites ne comprennent ni génome, ni ribosomes, mais contiennent certaines enzymes essentielles à différents processus cataboliques et anaboliques. Certaines de ces enzymes sont exprimées de manière continuelle tandis que d'autres sont induites dans certaines conditions appropriées.

Les peroxysomes effectuent un certain nombre de réactions essentielles telles que l'oxydation et la

respiration peroxysomale, la ß-oxydation des acides gras, le métabolisme du cholestérol et du dolichol, la synthèse des éthers phospholipides, le métabolisme du glyoxylate et le métabolisme de l'acide pipécolique.

Le métabolisme de l'oxydation peroxysomale comprend la formation de peroxyde d'hydrogène par un certain nombre d'oxydases et sa décomposition par une catalase.

Ces réactions sont responsables de la consommation de 20% de l'oxygène dans le foie. Différentes oxydases ont été identifiées dans les peroxysomes. L'élimination d'éthanol via la catalase dans le peroxysome et les processus d'oxydation via une déshydrogénase semblent être également des processus biochimiques importants.

Le système de ß-oxydation peroxysomale catalyse la ß-oxydation des chaînes courtes d'un certain nombre de dérivés d'acides gras qui ne peuvent être traités par les mitochondries. Celle-ci inclut l'oxydation des très longues chaînes d'acides gras, des acides di- ou trihydroxycholestanoïques, de l'acide pristanique, des longues chaînes d'acide dicarboxylique, de certaines prostaglandines, de certaines leukotriènes, des acides 12- et 15-hydroxyeicosatétraéonique, ainsi que de certains acides mono- et polyinsaturés. Le dosage des trois premiers composants est corrélé au diagnostic direct de certains désordres peroxysomaux.

Le peroxysome joue également un rôle essentiel dans la synthèse du cholestérol et d'autres isoprénoïdes. Les fibroblastes de patients affectés par un désordre de la biogenèse du peroxysome montrent une capacité inadéquate à synthétiser le cholestérol.

En outre, certains facteurs prolifératifs de peroxysomes provoquent une augmentation de l'incidence des tumeurs du foie dans certaines espèces.

Différents mécanismes ont été proposés pour la formation des hépato-carcinomes par des composants responsables de la prolifération peroxysomale, combinée à l'induction d'un stress oxydatif.

Par conséquent, l'identification de nouvelles molécules associées aux peroxysomes est d'une grande importance pour développer des outils diagnostiques et éventuellement des applications thérapeutiques dans le traitement de différentes maladies associées à des éventuelles déficiences de ces molécules.

De même, il est particulièrement utile d'identifier d'autres molécules présentes au niveau de certains organes, en particulier le poumon, et d'étudier leur association avec certaines pathologies, en particulier des maladies ou des lésions pulmonaires.

<u>Eléments caractéristiques de l'invention</u>

Les Inventeurs ont identifié chez l'homme, dans un lavage alvéolaire des poumons, un nouveau polypeptide dont la séquence nucléotidique et en acides aminés a été caractérisée. Ce nouveau polypeptide ou protéine a été dénommé protéine B18.

Cette molécule présente certaines homologies avec des protéines peroxysomales de la levure et possède un tripeptide carboxyterminal SQL connu pour cibler et faciliter la translocation de certaines protéines au niveau du peroxysome.

L'objet de la présente invention est relatif à toute séquence d'acides nucléiques présentant plus de

70%, avantageusement plus de 85%, de préférence plus de 95% d'homologie avec la séquence SEQ ID NO 1 ou son brin complémentaire décrits ci-après.

La présente invention concerne également la séquence nucléotidique SEQ ID NO 1, son brin complémentaire ou des portions de ceux-ci (figure 5).

On entend par "portions de la séquence SEQ ID NO 1", toute séquence nucléotidique de plus de 15 paires de base susceptible d'identifier ou reconstituer (de préférence par amplification génétique) la séquence SEQ ID NO 1. De telles méthodes d'identification ou de reconstitution sont basées sur la technique d'hybridation, de préférence dans des conditions stringentes, par des sondes marquées (par un élément radioactif, par une enzyme, par un marqueur fluorescent, etc.) ou sur la technique de l'amplification génétique par l'emploi d'une ou plusieurs amorces spécifiques d'au moins 15 nucléotides, permettant d'identifier la séquence SEQ ID NO 1 et/ou de la reconstituer par des techniques d'amplification génétique bien connues de l'homme de l'art, en particulier les technologies PCR, LCR, CPR, etc.

Un autre aspect de la présente invention concerne la séquence d'acides aminés encodée par les séquences nucléotidiques telles que définies ci-dessus.

La présente invention concerne également une séquence d'acides aminés présentant plus de 70%, avantageusement plus de 85%, de préférence plus de 95%, d'homologie avec la séquence SEQ ID NO 1 (figure 5).

Un autre aspect de la présente invention est relatif à un polypeptide dont la séquence d'acides aminés est la séquence SEQ ID NO 1 ou une portion de celle-ci.

On entend par "portion de la séquence SEQ ID NO 1", un fragment de la séquence SEQ ID NO 1 ayant subi une ou plusieurs délétions tout en conservant plus de 85%, de préférence plus de 95%, de son activité biochimique.

De préférence, ladite séquence polypeptidique SEQ ID NO 1 possède un PI de 7.3 et un poids moléculaire de 17000 D, tels que définis après une électrophorèse bidimensionnelle.

L'invention concerne également les anticorps, y compris des fragments de ceux-ci tels que les extrémités hypervariables Fab, ..., dirigés contre la séquence nucléotidique et peptidique selon l'invention.

Un autre aspect de la présente invention concerne un dispositif de diagnostic tel qu'une trousse de diagnostic ou une colonne de chromatographie comprenant un élément choisi parmi le groupe constitué par les séquences nucléotidiques, les séquences d'acides aminés et/ou des fragments de celles-ci selon l'invention et tels que définis ci-dessus. Ledit dispositif de diagnostic peut également comprendre un ou plusieurs réactif pour la détection et/ou le dosage de séquences nucléotidiques et/ou polypeptidiques basées sur les méthodes choisies parmi le groupe constitué par l'hybridation in situ, l'hybridation et/ou la reconnaissance par anticorps marqués, en particulier la technologie ELISA (Enzymes Linked Immuno-Sorbent Assay) ou RIA (Radio Immuno Assay), la détection sur filtre, sur support solide, en solution, en sandwich, en gel, par hybridation dot blot, Northern blot, Southern blot, marquage isotopique ou non isotopique (en particulier l'immunofluorescence ou la biotinilisation), la technique d'amplification génétique, la technique de double immunodiffusion, la technique de contre-électrophorèse, la

celles-ci) dans la composition pharmaceutique peut varier selon de très larges gammes, uniquement limitées par la fréquence d'administration, la tolérance et le niveau d'acceptation de la composition selon l'invention par le patient.

Un dernier aspect de la présente invention concerne l'utilisation du dispositif de diagnostic selon l'invention pour le diagnostic de maladies et/ou de lésions physiologiques chez l'homme ou l'animal, en particulier pour le diagnostic de maladies et/ou de lésions pulmonaires.

La présente invention concerne également l'utilisation de la composition pharmaceutique selon l'invention pour la préparation d'un médicament destiné au traitement et/ou à la prévention de maladies et/ou de lésions physiologiques chez l'homme ou l'animal, en particulier de maladies et/ou de lésions pulmonaires.

La présente invention sera décrite en détails dans les exemples suivants, en référence aux figures annexées.

Brève description des figures

La figure 1 représente une analyse par Northern blot de la présence de l'ARN messager encodant le polypeptide selon l'invention dans différents types de tissus humains.

La figure 2 représente une analyse par Northern blot de la présence de l'ARN messager encodant le polypeptide selon l'invention au niveau du poumon d'un rat après administration de lipopolysaccharides (LPS) induisant une réaction inflammatoire du poumon.

La figure 3 représente une analyse par Northern blot de la présence de l'ARN messager encodant le polypeptide B18 selon l'invention au niveau du poumon d'un rat après une injection intra-péritonéale d'agents pneumotoxiques.

La figure 4 représente l'alignement de la séquence nucléotidique selon l'invention avec une séquence nucléotidique connue (U82615).

La figure 5 représente la séquence nucléotidique SEQ ID NO 1 selon l'invention.

Exemple 1 : Homologie de la séquence SEQ ID NO 1 avec des séquences connues

Le polypeptide B18 (figures 5 et 6) de l'invention a été aligné avec des séquences homologues de protéines connues présentes dans les banques de données (GenBank, EMBL, DDBJ, PDB) ainsi qu'avec des séquences EST présentes dans GenBank. Ces résultats sont repris dans le tableau 1 ci-dessous.

Tableau 1 : Homologies entre la protéine B18 (162 acides aminés) et d'autres protéines

Nom	Code identification NCBI ID	Identité (% d'homologie)
Protéine de membrane (synechocystis sp.)	1652859	33/60 (55%) 8/19 (42%) 9/23 (39%)
Lipomyces kononenkoae putative peroxisomal protein	558080	32/75 (42%) 7/23 (30%) 7/18 (43%)

Nom	Code identification NCBI ID	Id ntité (% d'homologie)
Protéine hypothétique Haein HI0572	1723174	32/76 (42%) 10/26 (38%)
Non connu (Rhizobium sp.)	1486441	31/61 (50%) 8/20 (40%)
Protéine A de la membrane peroxysomale (PMP20)	130360	29/69 (42%) 8/14 (57%)
Protéine D de la membrane peroxysomale (PMP20)	130361	30/82 (36%) 8/14 (57%)
Protéine peroxysomale PMP yeast putative	1709682	12/33 (36%) 8/28 (28%) 7/11 (63%)
Protéine alkylhydroperoxyde réductase	1591451	14/44 (38%) 8/28 (28%)

Le tableau 2 reprend les pourcentages d'homologie entre le cDNA de la protéine B18 (805 nucléotides) avec d'autres séquences nucléotidiques.

5

Tableau 2

Nom	Numéro d'accès	Identité
mRNA humain sous la régulation de cellules infectées par des adenovirus 5	U82616	273/292 (93%)
	U82615	129/136 (94%) 99/108 (91%) 74/105 (70%)

La figure 4 représente l'alignement de la séquence nucléotidique du B18 et de la séquence

nucléotidique U82615 du tableau 2.

Le listing ci-dessous reprend les séquences EST présentant une homologie avec le cDNA de la protéine B18 (805 nucléotides).

Humain :

AA130751, N42215, W38597, N91311, N68467, AA187737, N68916, W00593, R88950, AA181884, H20154, H66666

Souris :

AA220019, AA123351, AA087129, AA255021, AA249897, W71344

Exemple 2

Une analyse par Northern blot dans différents tissus humains de l'ARN messager encodant le polypeptide B18 humain selon l'invention et représentée sur la figure 1 annexée donne une révélation particulière au niveau du poumon.

Cette analyse a été obtenue à partir d'une trousse Multiple Tissues Northern Blot ® (Clontech), comprenant approximativement 2 µg d'une séquence poly-A et d'un ARN humain dans chacune des lignes hybridées avec une sonde B18 de 554 paires de bases fixée et reportée avec une sonde de ß-actine de 2 kb; toutes deux marquées à l'élément radioactif ^{32}P.

L'analyse par Northern blot a été déterminée par Phosphorimaging Technology et les données relatives au mRNA du polypeptide B18 ont été normalisées par rapport au niveau de mRNA de la ß-actine.

Exemple 3

Une analyse par Northern blot du mRNA codant pour la protéine B18 du rat a été analysée au niveau du poumon du rat après 6, 48 et 72 heures suivant l'administration au rat de lipopolysaccharides (LPS) induisant une réaction inflammatoire au niveau du poumon.

Cette analyse a été obtenue par Nothern Blot au moyen de 15 µg de RNA total hybridé sur chaque bande avec une sonde de 225 paires de base encodant la protéine B18 du rat, fixée et reportée sur une sonde de 572 paires de base encodant la ß-actine du rat; les deux sondes étant marquées à l'élément radioactif ^{32}P.

L'analyse par Northern blot a été quantifiée par Phosphorimaging Technology et les données relatives au mRNA encodant le polypeptide B18 ont été normalisées par rapport au niveau de mRNA de la ß-actine.

La figure 2 indique que l'on peut utiliser les séquences selon l'invention comme marqueurs d'une infection inflammatoire au niveau des poumons.

Exemple 4

Les Inventeurs ont fait une analyse par Northern blot de la présence de mRNA encodant le polypeptide B18 au niveau d'un poumon de rat après injection intra-péritonéale d'agents pneumotoxiques.

Ces agents sont le 4-ipoméanol,1-(3-fyryl)-4-hydroxypentanone (IPO), le méthylcyclopentadiényle manganèse tricarbonyle (MMT) et le α-naphtylthiourée (ANTU).

Ces agents sont connus pour induire au niveau des poumons des lésions aiguës des cellules de clara (IPO) ou des lésions aiguës au niveau des cellules alvéolaires

(MMT) ainsi que pour provoquer une augmentation de la perméabilité de la barrière sanguine au niveau des alvéoles (ANTU).

L'analyse par Northern blot a été effectuée au moyen de 15 µg de RNA total hybridé sur chaque bande avec une sonde de 225 paires de base encodant le polypeptide B18 du rat, fixée et reportée sur une sonde de 572 paires de base encodant la ß-actine du rat; les deux sondes étant marquées à l'élément radioactif ^{32}P.

L'analyse par Northern blot a été quantifiée par la Phosphorimaging Technology et les données relatives au mRNA du polypeptide B18 ont été normalisées par rapport au niveau de mRNA de la ß-actine.

La figure 3 montre que les séquences nucléotidiques selon l'invention peuvent être utilisées comme marqueurs de lésions induites par l'injection d'agents pneumotoxiques connus ou non connus.

REVENDICATIONS

1. Séquence d'acides nucléiques présentant plus de 70% d'homologie avec la séquence SEQ ID NO 1 ou son brin complémentaire.

2. Séquence d'acides nucléiques présentant plus de 85% d'homologie avec la séquence SEQ ID NO 1 ou son brin complémentaire.

3. Séquence d'acides nucléiques présentant plus de 95% d'homologie avec la séquence SEQ ID NO 1 ou son brin complémentaire.

4. Séquence d'acides nucléiques correspondant à la séquence SEQ ID NO 1, son brin complémentaire ou des portions de ceux-ci comprenant plus de 15 paires de base, susceptible d'identifier ou de reconstituer la séquence SEQ ID NO 1.

5. Séquence d'acides aminés présentant plus de 70% d'homologie avec la séquence SEQ ID NO 1.

6. Séquence d'acides aminés présentant plus de 85% d'homologie avec la séquence SEQ ID NO 1.

7. Séquence d'acides aminés présentant plus de 95% d'homologie avec la séquence SEQ ID NO 1.

8. Séquence d'acides aminés correspondant à la séquence SEQ ID NO 1 ou une portion de celle-ci.

9. Anticorps dirigé contre les séquences selon l'une quelconque des revendications précédentes.

10. Dispositif de diagnostic comprenant un élément choisi parmi le groupe constitué par les séquences d'acides nucléiques, les séquences d'acides aminés, des portions de celles-ci et/ou les anticorps selon l'une quelconque des revendications précédentes.

11. Dispositif selon la revendication 10, caractérisé en ce qu'il est une trousse de diagnostic ou

une colonne de chromatographie.

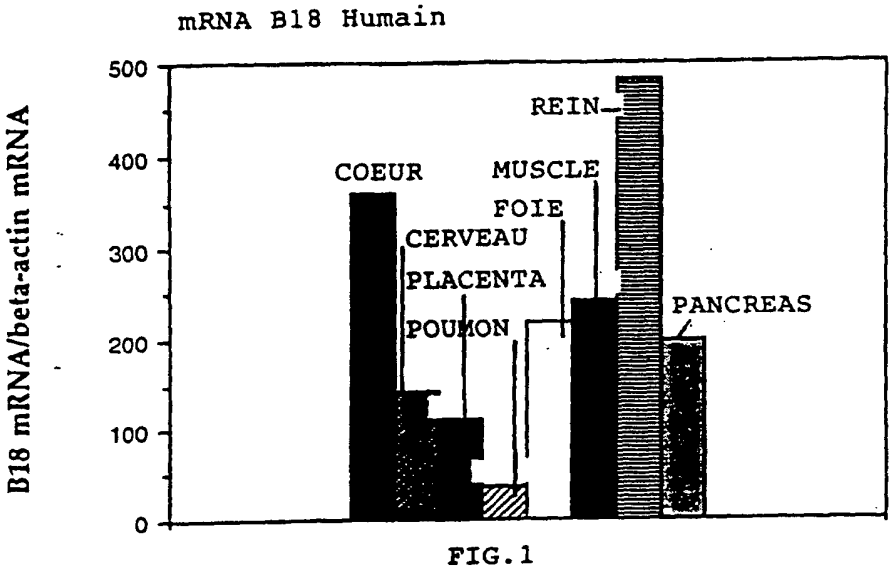
mRNA B18 Humain
B18 mRNA/beta-actin mRNA
500
400
300
200
100
0
COEUR
CERVEAU
PLACENTA
POUMON
FOIE
MUSCLE
REIN
PANCREAS

FIG.1

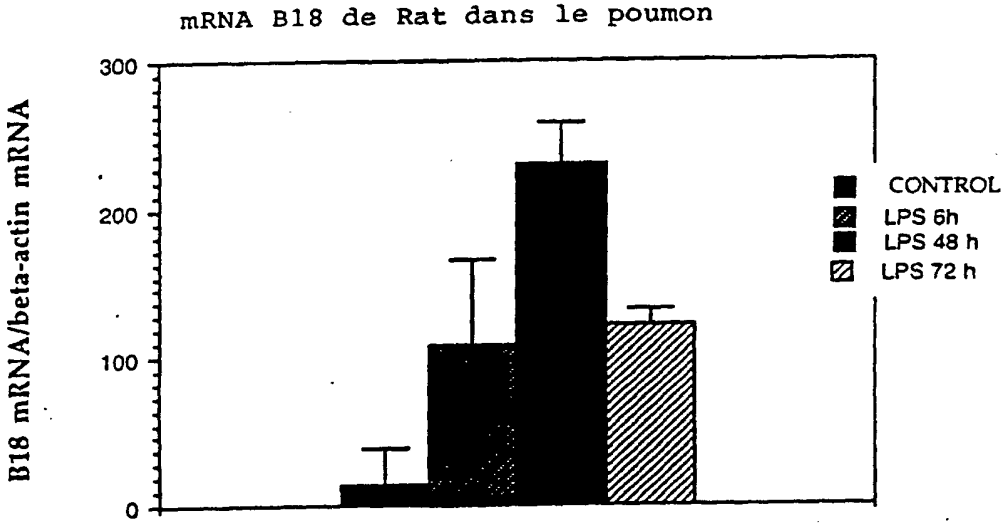
mRNA B18 de Rat dans le poumon
B18 mRNA/beta-actin mRNA
300
200
100
0
CONTROL
LPS 6h
LPS 48 h
LPS 72 h

FIG.2

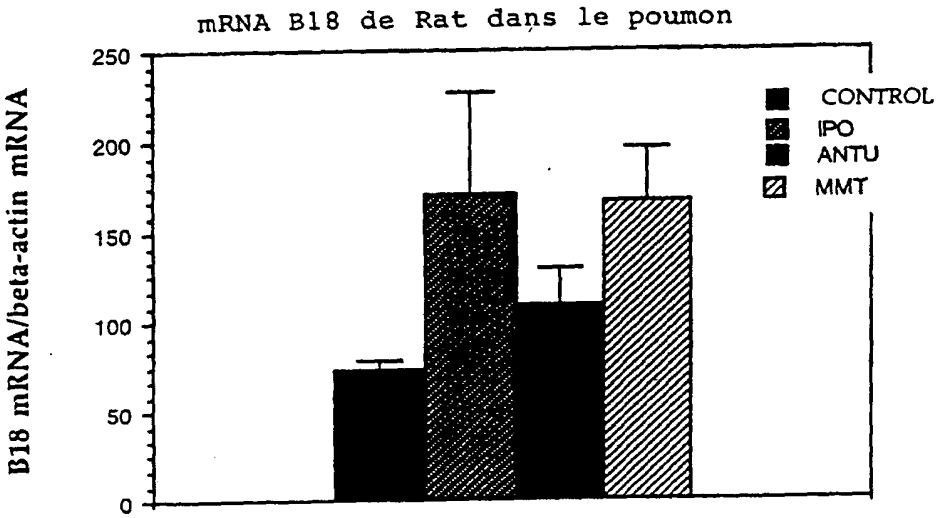
mRNA B18 de Rat dans le poumon
B18 mRNA/beta-actin mRNA
250
200
150
100
50
0
CONTROL
IPO
ANTU
MMT

FIG.3

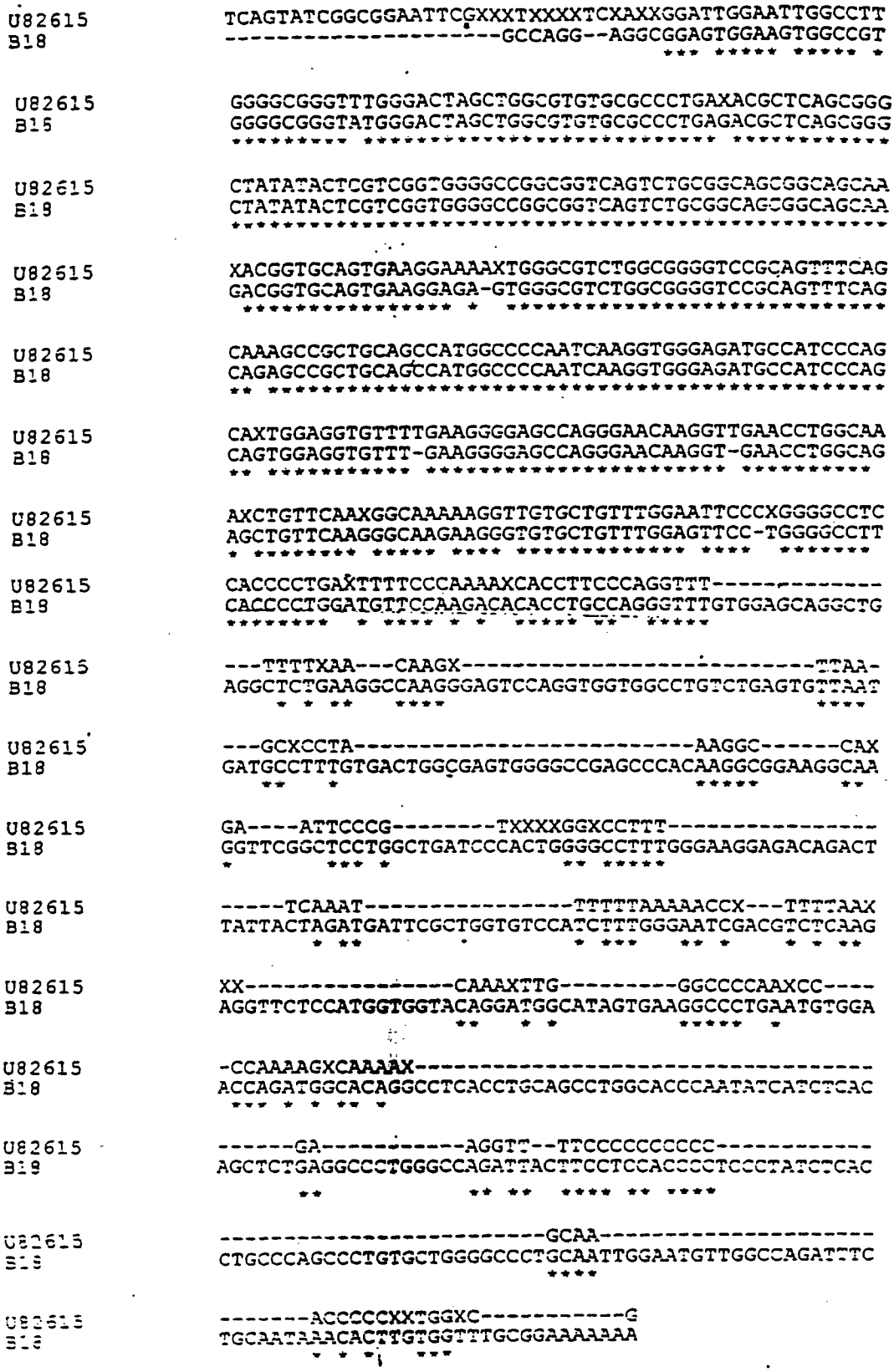
U82615
B18
TCAGTATCGGCGGAATTCGXXXTXXXXTCXAXXGGATTGGAATTGGCCTT
----------GCCAGG--AGGCGGAGTGGAAGTGGCCGT
U82615
B18
GGGGCGGGTTTGGGACTAGCTGGCGTGTGCGCCCTGAXACGCTCAGCGGG
GGGGCGGGTATGGGACTAGCTGGCGTGTGCGCCCTGAGACGCTCAGCGGG
U82615
B18
CTATATACTCGTCGGTGGGGCCGGCGGTCAGTCTGCGGCAGCGGCAGCAA
CTATATACTCGTCGGTGGGGCCGGCGGTCAGTCTGCGGCAGCGGCAGCAA
U82615
B18
XACGGTGCAGTGAAGGAAAAXTGGGCGTCTGGCGGGGTCCGCAGTTTCAG
GACGGTGCAGTGAAGGAGA-GTGGGCGTCTGGCGGGGTCCGCAGTTTCAG
U82615
B18
CAAAGCCGCTGCAGCCATGGCCCCAATCAAGGTGGGAGATGCCATCCCAG
CAGAGCCGCTGCAGCCATGGCCCCAATCAAGGTGGGAGATGCCATCCCAG
U82615
B18
CAXTGGAGGTGTTTTGAAGGGGAGCCAGGGAACAAGGTTGAACCTGGCAA
CAGTGGAGGTGTTT-GAAGGGGAGCCAGGGAACAAGGT-GAACCTGGCAG
U82615
B18
AXCTGTTCAAXGGCAAAAAGGTTGTGCTGTTTGGAATTCCCXGGGGCCTC
AGCTGTTCAAGGGCAAGAAGGGTGTGCTGTTTGGAGTTCC-TGGGGCCTT
U82615
B18
CACCCCTGAXTTTTCCCAAAAXCACCTTCCCAGGTTT----------
CACCCCTGGATGTTCCAAGACACACCTGCCAGGGTTTGTGGAGCAGGCTG
U82615
B18
---TTTTXAA---CAAGX----------TTAA-
AGGCTCTGAAGGCCAAGGGAGTCCAGGTGGTGGCCTGTCTGAGTGTTAAT
U82615
B18
---GCXCCTA----------AAGGC------CAX
GATGCCTTTGTGACTGGCGAGTGGGGCCGAGCCCACAAGGCGGAAGGCAA
U82615
B18
GA----ATTCCCG--------TXXXXGGXCCTTT----------
GGTTCGGCTCCTGGCTGATCCCACTGGGGCCTTTGGGAAGGAGACAGACT
U82615
B18
-----TCAAAT----------TTTTTAAAAACCX---TTTTAAX
TATTACTAGATGATTCGCTGGTGTCCATCTTTGGGAATCGACGTCTCAAG
U82615
B18
XX----------CAAAXTTG---------GGCCCCAAXCC----
AGGTTCTCCATGGTGGTACAGGATGGCATAGTGAAGGCCCTGAATGTGGA
U82615
B18
-CCAAAAGXCAAAAX----------
ACCAGATGGCACAGGCCTCACCTGCAGCCTGGCACCCAATATCATCTCAC
U82615
B18
------GA----------AGGTT--TTCCCCCCCCCC----------
AGCTCTGAGGCCCTGGGCCAGATTACTTCCTCCACCCCTCCCTATCTCAC
U82615
B18
----------GCAA----------
CTGCCCAGCCCTGTGCTGGGGCCCTGCAATTGGAATGTTGGCCAGATTTC
U82615
B18
-------ACCCCCXXTGGXC----------G
TGCAATAAACACTTGTGGTTTGCGGAAAAAAA

FIG. 4

Sequence B18 Protéine: pI (théorique): 6,96-7,3 MW: 16.899
aa: 162

```
                                                                    60
GCCAGGAGGCGGAGTGGAAGTGGCCGTGGGGCGGGTATGGGACTAGCTGGCGTGTGCGCC

                                                                   120
CTGAGACGCTCAGCGGGCTATATACTCGTCGGTGGGGCCGGCGGTCAGTCTGCGGCAGCG

                                                                   180
GCAGCAAGACGGTGCAGTGAAGGAGAGTGGGCGTCTGGCGGGGTCCGCAGTTTCAGCAGA

                                                                   240
GCCGCTGCAGCCATGGCCCCAATCAAGGTGGGAGATGCCATCCCAGCAGTGGAGGTGTTT
              M  A  P  I  K  V  G  D  A  I  P  A  V  E  V  F

                                                                   300
GAAGGGGAGCCAGGGAACAAGGTGAACCTGGCAGAGCTGTTCAAGGGCAAGAAGGGTGTG
 E  G  E  P  G  N  K  V  N  L  A  E  L  F  K  G  K  K  G  V

                                                                   360
CTGTTTGGAGTTCCTGGGGCCTTCACCCCTGGATGTTCCAAGACACACCTGCCAGGGTTT
 L  F  G  V  P  G  A  F  T  P  G  C  S  K  T  H  L  P  G  F

                                                                   420
GTGGAGCAGGCTGAGGCTCTGAAGGCCAAGGGAGTCCAGGTGGTGGCCTGTCTGAGTGTT
 V  E  Q  A  E  A  L  K  A  K  G  V  Q  V  V  A  C  L  S  V

                                                                   480
AATGATGCCTTTGTGACTGGCGAGTGGGGCCGAGCCCACAAGGCGGAAGGCAAGGTTCGG
 N  D  A  F  V  T  G  E  W  G  R  A  H  K  A  E  G  K  V  R

                                                                   540
CTCCTGGCTGATCCCACTGGGGCCTTTGGGAAGGAGACAGACTTATTACTAGATGATTCG
 L  L  A  D  P  T  G  A  F  G  K  E  T  D  L  L  L  D  D  S

                                                                   600
CTGGTGTCCATCTTTGGGAATCGACGTCTCAAGAGGTTCTCCATGGTGGTACAGGATGGC
 L  V  S  I  F  G  N  R  R  L  K  R  F  S  M  V  V  Q  D  G

                                                                   660
ATAGTGAAGGCCCTGAATGTGGAACCAGATGGCACAGGCCTCACCTGCAGCCTGGCACCC
 I  V  K  A  L  N  V  E  P  D  G  T  G  L  T  C  S  L  A  P

                                                                   720
AATATCATCTCACAGCTCTGAGGCCCTGGGCCAGATTACTTCCTCCACCCCTCCCTATCT
 N  I  I  S  Q  L  *

                                                                   780
CACCTGCCCAGCCCTGTGCTGGGGCCCTGCAATTGGAATGTTGGCCAGATTTCTGCAATA


                                                                   840
AACACTTGTGGTTTGCGGAAAAAAA
```

FIG.5

Annexe 2

Running title : overexpression of PRDX5 in CHO cells

Overexpression of human peroxiredoxin 5 in Chinese Hamster Ovary cells: effects on cell survival and DNA damage during acute oxidative stress induced by peroxides*

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The abbreviations used are: ANT, adenine nucleotide translocase; CHO, chinese hamster ovary cells; GPX, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; H_2O_2, hydrogen peroxide; MPT, mitochondrial transition permeability; NLS, nuclear localization sequence; $O_2^{\cdot-}$, superoxide anion; $^{\cdot}OH$, hydroxyl radical; PRDX, peroxiredoxin; PTS, peroxisomal targeting signal; $RO^{\cdot}$, alkoxyl radical; $ROO^{\cdot}$, peroxyl radical; ROS, reactive oxygen species; TBARS, thiobarbituric acid-reactive species; *t*BHP, *tert*-butylhydroperoxide; TNF-α : tumor necrosis factor α.

Summary

Peroxiredoxins (PRDXs) are a family of peroxidases present in organisms from all kingdoms. PRDXs are able to reduce hydrogen peroxide as well as alkyl hydroperoxides with the use of reducing equivalents derived from thiol-containing donor molecules. Peroxiredoxin 5 (PRDX5) is a mammalian thioredoxin peroxidase ubiquitously expressed in tissues and intracellularly localized to mitochondria, peroxisomes, the cytosol and, to a less extent, to the nucleus. This remarkably wide subcellular distribution compared to the five other members of mammalian PRDXs prompted us to further investigate the antioxidant protective function of PRDX5 in mammalian cells according to its subcellular localization. Chinese hamster ovary cells overexpressing human PRDX5 in the cytosol, in mitochondria or in the nucleus were established by stable transfection. Cells overexpressing peroxiredoxin 5 were exposed to acute oxidative stress with hydrogen peroxide (20-40 mM) or *tert*-butylhydroperoxide (20-70 mM) during an hour and viability was evaluated by lactate dehydrogenase assay. Overexpressing PRDX5 in either cytosolic or mitochondrial compartments significantly reduced cell death with a more effective protection with overexpression of PRDX5 in mitochondria confirming that this organelle is a main target for peroxides. Moreover, we evaluated DNA damage induced by sublethal concentrations of hydrogen peroxide (2-5 mM) or *tert*-butylhydroperoxide (5-10 mM) with the comet assay. Overexpression of PRDX5 in the nucleus significantly decreased DNA damage induced by both peroxides. Taken together, our results show that overexpression of PRDX5 by Chinese hamster ovary cells in cytosolic and mitochondrial compartments increases cell survival of cells challenged by peroxides and that overexpression of PRDX5 in the nucleus decreases DNA damages induced by peroxides. These results suggest that PRDX5 could be implicated in general antioxidant defenses in

mammalian cells during acute oxidative stress such as inflammation.

Introduction

Reactive oxygen species (ROS) are constantly generated under normal conditions as a consequence of aerobic metabolism. ROS include free radicals such as the superoxide anion ($O_2^{\cdot-}$), the hydroxyl radical ($^{\cdot}OH$) and the non-radical hydrogen peroxide (H_2O_2). They are particularly transient species due to their chemical reactivity and can react with DNA, proteins and lipids in a destructive manner (1). To protect themselves from the deleterious effects of reactive oxygen species, cells have developed a wide range of antioxidant systems to limit the production of reactive oxygen species or to inactivate them. However, oxidative stress may occur when the balance between reactive oxygen species production and antioxidant defense is disrupted.

Over a decade ago, a novel family of peroxidases named peroxiredoxins (PRDXs) has emerged (2). PRDXs are a family of peroxidases well conserved from bacteria to humans. Six members (PRDX1 to 6) have been identified in mammals (reviewed in 3 and 4). These enzymes have distinct tissue distribution profiles and different subcellular localizations. All these enzymes have been described as peroxidases, which reduce hydrogen peroxide and organic hydroperoxides using electrons donated by thioredoxin (PRDX1 to 5) or glutathione (PRDX6) (reviewed in 4).

Recently, we have cloned and characterized PRDX5 (5) also known as PrxV/PMP20/AOEB166/ARC1 (5-8). PRDX5 is expressed in many tissues in mammals (3, 5). Moreover, this enzyme has the largest intracellular localization amongst PRDXs. Indeed, identified mitochondrial and peroxisomal targeting sequences address PRDX5 to these organelles (5, 7). PRDX5 has also a cytosolic localization (6) and, despite the

lack of a clear nuclear localization signal, PRDX5 has been reported to be localized in the nucleus of epithelial cells in rat kidney (9). The ubiquitous distribution and high levels of PRDX5 in tissues suggest that it might play an important antioxidant role in organelles that are the major sources of ROS namely peroxisomes and mitochondria. Moreover, the peroxidase function of PRDX5 in cells has been demonstrated by the observation that transient expression of the wild-type protein, unlike the N-terminal cysteine mutant, in NIH3T3 cells inhibited hydrogen peroxide accumulation induced by TNF-α (6). Moreover, transient overexpression of PRDX5 in Cos-7 cells decreased apoptotic death induced by staurosporine and *tert*-butylhydroperoxide (*t*BHP) (10). Finally, Zhou *et al.* (11) reported that PRDX5 prevented the p53-dependent generation of ROS.

The purpose of this article was to further analyze the functional significance of PRDX5 as an antioxidant protective enzyme during acute oxidative stress according to its subcellular localization. PRDX5 was stably overexpressed in Chinese hamster ovary (CHO) cells in different subcellular compartments: in the cytosol, in mitochondria and in the nucleus. To specifically target PRDX5 to the nucleus, the SV40 Large-T antigen nuclear localization sequence was fused to the amino-terminus. The influence of overexpressing PRDX5 in CHO cells on catalase and glutathione peroxidase activities, antioxidant enzymes that have similar localizations and activities was also examined. Then, evaluation of antioxidant protection conferred by PRDX5 was performed. Stably transfected cell lines were submitted to acute oxidative stress induced by hydrogen peroxide or *tert*-butylhydroperoxide, and cytotoxicity was evaluated. As PRDX5 was also localized in the nucleus, we investigated the protective effects of PRDX5 on DNA against genotoxic concentrations of peroxides.

Experimental procedures

Materials - CHO-K1 cells were kindly provided by Dr. Yves-Jacques Schneider (Catholic University of Louvain). Plasmid pEF-BOS was kindly provided by Dr. Jean-Christophe Renauld (Catholic University of Louvain). Specific polyclonal antibodies directed to human PRDX5 were obtained as described before (12). Hydrogen peroxide, *tert*-butylhydroperoxyde (*t*BHP), glutathione peroxidase, NADPH, rabbit anti-actin, 4,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma Chemical Co. Glutathione, glutathione reductase, the Cytotoxicity Detection Kit (LDH) and FuGENE-6 were obtained from Roche. The BCA protein assay reagent was from Pierce. Fetal bovine serum, Dulbecco's modified Eagle's media, penicillin, streptomycin and trypsin-EDTA were purchased from Life Technologies, Inc. Peroxidase-conjugated goat anti-rabbit IgG were from Dako and FITC-conjugated donkey anti-rabbit IgG from Jackson. The Western Lightning chemiluminescence reagent plus was obtained from PerkinElmer Life Sciences Inc.

Cell culture - CHO-K1 cells were cultivated in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin (completed DMEM) in a humidified atmosphere, in 5 % CO_2 at 37 °C. Cells were passaged at a 1:5 ratio every 3 days with 0.05% trypsin-EDTA.

Plasmids construction - Plasmid pEF-BOS (13, 14) with an EF-1-α promoter and G-CSF-poly(A) adenylation signal was used as a mammalian expression vector. For the construction of pEF-BOS-Mito-PRDX5 (PRDX5 cDNA sequence containing the mitochondrial targeting sequence), human PRDX5 cDNA (Knoops *et al.*, 1999) was amplified by PCR using forward primer 5'-GGCCGTGAATTCGGTATGGGACTAGCTGGC-3' (EcoRI site underlined) and

reverse primer 5'-TAATCTGCGGCCGCGCCTCAGAGCTGTGAGAT-3' (NotI site underlined), cloned into EcoRI and NotI sites of pCMS-EGFP (Clontech), subcloned into EcoRI and NotI sites of pTracer-SV40 (Invitrogen) and finally cloned into SpeI and NotI sites of pEF-BOS. For the construction of pEF-BOS-Cyto-PRDX5 (short coding sequence of human PRDX5 without its mitochondrial sequence) the procedure was as before for the construct of pEF-BOS-Mito-PRDX5 except that the forward primer was 5'-AGAGCCGAATTCGCCATGGCCCCAATCAAG-3' (EcoRI site underlined). To target PRDX5 to the nucleus, the SV40 Large-T antigen nuclear localization sequence (NLS) was fused to the N-terminus of the short PRDX5 (Cyto-PRDX5). Primers 5'-GCGTCTACTAGTGCC*ATG*GGG**CCAAAGAAGAAGCGAAAGGTC**GGGGCCCCAAT CAAGGTGGGAGATG-3' (SpeI site underlined; start codon in italic; NLS coding sequence in bold surrounded by two glycine codons) and 5'-TAATCTGCGGCCGCGCCTCAGAGCTGTGAGATGATATTG-3' (NotI site underlined) were used to amplify PRDX5 cDNA. The resulting amplicon was ligated into the NotI-SpeI site of pEF-BOS vector generating pEF-BOS-Nuc-PRDX5.

Stable transfection of CHO cells - Before transfection, 3.10^5 CHO cells were seeded into 6-well plates and grown to 50-80 % confluency. Plasmids pEF-BOS, pEF-BOS-Mito-PRDX5, pEF-BOS-Cyto-PRDX5 and pEF-BOS-Nuc-PRDX5 were transfected into CHO cells using the FuGENE-6 transfection reagent according to the instructions provided by the manufacturer. Selection for puromycin resistance (4 µg/ml) was initiated forty-eight hours after transfection, and cells were continually exposed to puromycin (4 µg/ml). Individual clones were isolated by dilution technique to obtain stably transfected cell lines. Overexpression of PRDX5 was verified by immunofluorescence (see below immunostaining).

Glutathione peroxidase activities - Fresh sonicated extracts from $5\text{-}10.10^7$ CHO cells

were used. Glutathione peroxidase activity was assayed by using GSH and *tert*-butylhydroperoxide as substrates and was monitored by GSSG production through NADPH oxidation by glutathione reductase, according to Paglia and Valentine (15). GPX activities were estimated from a standard curve constructed with GPX purified from bovine erythrocytes and were expressed as nmol of NADPH x min^{-1} x mg^{-1} protein.

Catalase activity - Fresh sonicated extracts from 5-10.10^7 cells were used. Ten to twenty µg of total cellular protein were used. Catalase activities were determined at 0°C as described by Rickwood and Harris (16). This colorimetric method allows the indirect quantification of H_2O_2 by the formation of a peroxo-titanic complex that can be detected at 405 nm. Specific activity (units x min^{-1} x mg^{-1} protein) was calculated from the equation: log $(Abs_1 - Abs\ Blank_1)/(Abs_2 - Abs\ Blank_2)$ x reaction volume x time $(min)^{-1}$ x mg^{-1} protein where Abs_1 is the absorbance of the buffer with hydrogen peroxide, $Abs\ Blank_1$ is the absorbance of the buffer, Abs_2 is the absorbance of the buffer with lysat and hydrogen peroxide, $Abs\ Blank_2$ is the absorbance of the buffer and lysat.

Immunostaining - Cells were cultivated on coverslips into 24-well plates in 1 ml supplemented DMEM medium containing puromycin (4µg/ml). After 15 to 18 hours, cells at 70 % confluency were fixed for 30 min on coverslips with 4 % formaldehyde. For immunostaining, the procedure described by Knoops *et al.* (5) was followed. Cells were incubated sequentially overnight with 1:200 rabbit anti-human PRDX5 polyclonal antibody (12) and one hour with 1:50 FITC-conjugated donkey anti-rabbit IgG. The coverslips were mounted in Mowiol containing 50 µg/ml DAPI (4,6-diamidino-2-phenylindole) and examined by fluorescence microscopy with appropriate filters.

Western Blotting - To determine total cellular PRDX5 or β-actin protein levels, cells

were cultivated in 75 cm^2 culture flasks. When 70-90 % confluency was reached, cells were washed with PBS, harvested by trypsinisation and sonicated. The sonicated suspensions were centrifuged at 10,000 x g for 10 min at 4°C. The supernatant was removed and the protein concentration was determined. Thirty µg of denaturated protein from the supernatant were resolved on 12 % SDS-PAGE and electroblotted onto nitrocellulose membranes. Membranes were incubated overnight with 1:1000 rabbit anti-human PRDX5 polyclonal antibody or with 1:200 rabbit anti-actin, followed by incubation for 1 hour with 1:5000 peroxidase-conjugated goat anti-rabbit IgG. Detection by the chemiluminescence reaction was carried out by using the Western Lightning chemiluminescence reagent plus, followed by exposure to Hyperfilm (Amersham Pharmacia). Quantification was made by densitometric analyzes (Kodak Image Station) of the intensity of the various bands. Normalization of PRDX5 protein levels was performed with β-actin levels.

Treatment of cells with hydrogen peroxide and tBHP - Exponentially growing cells were resuspended in fresh medium and were seeded at a density of 3.10^4 cells/cm^2 into 12-well plates (for cytotoxicity test) or into 6-well plates (for comet assay). After 15-18 hours of culture, stress was induced on 70-90 % confluent cells in serum-free DMEM medium without phenol red (37°C, 95% humidity, 5% CO_2).

Lactate Dehydrogenase (LDH) Assay - Cytotoxicity was evaluated by the lactate dehydrogenase release assay (Cytotoxicity Detection Kit (LDH), Roche). One hundred microliters of cellular supernatants from treated and untreated cells were used for the LDH assay. The total LDH release (100 % cell death) was determined for each CHO clone after lysis of cells with 2 % Triton X-100 detergent. The mortality of cells was calculated according to this scale.

Lipid peroxidation assay - The extent of lipid peroxidation was determined by quantifying the amount of TBARS in the medium. Treatments were carried out in 6-well plates containing serum-free DMEM medium without phenol red, as it could interfere with the assay. TBARS released into the culture medium were assayed as described by Dubuisson *et al.* (17). Standard curves specific for the assays were created using malondialdehyde (MDA) and the signal converted into MDA equivalents x mg^{-1} protein.

Comet assay - Cell treated appropriately with peroxides were washed twice with PBS. The monolayer was detached by trypsinisation, and cells were collected and embedded in 1 % low-melting-point agarose. The comet assay was performed according to the method of Collins *et al.* (18). CHO nucleoids were analyzed with a fluorescence microscope equipped with a 362 nm (exc.) monochromator. Three slides were scored by computerized image analysis of 50 randomly selected comets per slide using Komet 4.0.2 (Kinetic Imaging, Ltd., Liverpool, UK). Percentage of tail DNA was chosen to evaluate the frequency of break DNA damages.

Quantification of proteins - Protein content was determined with the BCA protein assay reagent (Pierce) with Bovine Serum Albumine as the standard.

Statistical analysis - Means and standard errors (SEM) were calculated from replicates within an experiment, and each experiment was repeated three times. Statistical analysis was performed using one-way ANOVA followed by Newman-Keuls post-hoc test.

Results

Generation of CHO Cell Lines Overexpressing PRDX5 in the Cytosol, in Mitochondria and in the Nucleus - The human PRDX5 cDNA contains two ATG initiation codons (5, 6), giving a long or a short PRDX5 form. The short form contains a peroxisomal targeting signal type 1 (PTS 1) and is localized to peroxisomes, the cytosol and the nucleus, while the long form has a mitochondrial localization, due to the presence of a mitochondrial targeting sequence (MTS) which is a targeting sequence stronger than the PTS 1 (N.T. Nguyen-Nhu et al., in preparation). Fig. 1A represents the expression vector pEF-BOS with the PRDX5 construct. The promoter EF1-α allows a high expression of protein in mammalian cells. To target PRDX5 to the cytosol, mitochondria and the nucleus only, three different expression vectors were constructed (Fig.1B): pEF-BOS-Cyto-PRDX5 (1) contains the short form of PRDX5; pEF-BOS-Mito-PRDX5 (2) contains the long form of PRDX5 and pEF-BOS-Nuc-PRDX5 (3) contains the short form of PRDX5 in fusion with the SV40 Large-T antigen nuclear localization sequence (NLS) at the N-terminus. Chinese hamster ovary K-1 cells were transfected with the empty expression vector pEF-BOS or with one of the three PRDX5 expression vectors. Stable clones were selected upon puromycin resistance giving four cell lines named 'Control clone' transfected with empty expression vector, 'Cyto clone' overexpressing PRDX5 in the cytosol, peroxisomes, and the nucleus, 'Mito clone' overexpressing PRDX5 in mitochondria or 'Nuc clone' overexpressing PRDX5 in the nucleus.

Subcellular Localization of PRDX5 in CHO Cell Lines Overexpressing PRDX5 - By immunocytochemistry, the intracellular localization of PRDX5 in the four stable cell lines was determined (Fig. 2). In Control clone, a basal signal throughout cells was detected as well

as a typical mitochondrial reticular labelling. Cyto clone strongly expressed PRDX5 in the cytosol, as well as in the nucleus. Peroxisomes could not be visualized because of the intense cytosolic labelling but previous studies (5-7) ascertain the peroxisomal localization of the short PRDX5 form. In Mito clone, PRDX5 was strongly expressed in mitochondria, which appeared as a typical reticular or globular pattern. In Nuc clone, PRDX5 was strongly expressed in the nucleus.

Quantification of PRDX5 Overexpression in CHO Cell Lines - Overexpression of PRDX5 was quantified upon immunoblotting. Fig. 3A shows the expression of PRDX5 in the total cell extract from each clone, as determined by Western blot. Results from densitometry analyzes of the intensity of the various bands and after normalization of PRDX5 protein levels with β-actin are presented in Fig. 3B. The expression of PRDX5 in Cyto clone was 7.7-fold higher than the one in Control clone while in Mito clone, this expression was 3.4-fold higher and in Nuc clone, it was 5.9-fold higher.

Catalase and Glutathione Peroxidase Activities in CHO Cells Overexpressing PRDX5 - To determine if overexpression of PRDX5 modified the expression of other antioxidant enzymes, catalase and glutathione peroxidase activities were assessed. These antioxidant enzymes were chosen for their similarity with PRDX5 in cell localization (cytosolic, peroxisomal or mitochondrial) and activity (reduction of hydrogen and organic peroxides). As shown in Fig. 4, catalase activity was significantly higher in Cyto clone ($p < 0.01$) and in Mito clone ($p < 0.05$) as compared with Control clone, while Nuc clone had a normal catalase activity. In Fig. 5, glutathione peroxidase activity is represented. This activity was significantly higher in Cyto clone ($p < 0.05$), while in Mito and Nuc clones, there was no difference as compared with Control clone.

TBARS Content in PRDX5 Overexpressing CHO Cell Lines, in Absence of Stress - To determine if PRDX5 overexpression might induce some oxidative stress, TBARS measurements were realized from the four clones, in the absence of stress. No TBARS difference was detected from the different clones overexpressing PRDX5, as compared with Control clone (data not shown).

PRDX5 Overexpression Confers Cell Protection Against Oxidant Injury Induced by Hydrogen Peroxide and by Tert-butylhydroperoxide - To determine whether an increase in PRDX5 expression in different cell compartments could protect against oxidative stress induced by peroxides, the four clones were exposed to 20 mM or 40 mM H_2O_2 or to 20 mM or 70 mM *tert*-butylhydroperoxide (*t*BHP). As shown in Fig. 6A, the percentage of cell death increased with the concentration of H_2O_2. The Mito clone was the most resistant, with no cell death rise even at the highest dose of H_2O_2. Cyto clone also showed a good resistance. Indeed, the percentage of cell death at 40 mM was half reduced. Nuc clone showed no difference as compared with Control clone. Results obtained for *t*BHP are presented in Fig. 6B. The same observations as for H_2O_2 could be done. Mito clone showed a complete protection at both concentrations of *t*BHP; Cyto clone approximately abolished half of the cell death as compared to the Control clone. Expression of PRDX5 in the nucleus (Nuc clone) could not afford protection as compared with Control clone.

Nuclear PRDX5 Overexpression Confers DNA Protection against Oxidant Injury Induced by Hydrogen Peroxide and by Tert-butylhydroperoxide - DNA damage in the different transfected clones was then assessed in response to H_2O_2 and *t*BHP (Fig. 7). Comet assay was used and % of tail DNA was chosen to evaluate DNA damage. Stress was induced for one hour at non-cytotoxic concentrations of peroxides, determined by the LDH assay. At 2 or 5 mM, H_2O_2 induced 50 % of DNA damage (Fig. 7A). Nuc clone showed a lower % of

DNA damage at 2 mM of H_2O_2 ($p < 0.001$), while at 5 mM, there was no difference as compared with Control clone. Cyto and Mito clones showed no difference at both concentrations as compared with Control clone. *t*BHP induced nearly 50 % of DNA damage at 5 or 10 mM (Fig. 7B). As compared with Control clone, Nuc clone had reduced DNA damage at both concentrations, this reduction being more important at 5 mM (28 % DNA damage for Nuc clone and 47 % for Control clone) than at 10 mM (38 % DNA damage for Nuc clone and 48 % for Control clone). No decrease in DNA damage was observed at both concentrations of *t*BHP for Cyto and Mito clones.

Discussion

We have shown in this study that PRDX5 overexpression in the cytosol conferred a protection against H_2O_2. Interestingly, while overexpression of PRDX5 in mitochondria was weaker than in cytosol, as determined by immunoblots, it had a stronger protective effect. These results confirm that mitochondria might be the primary target for H_2O_2 even when H_2O_2 is exogenously applied to the cells. Furthermore, these results are in agreement with mechanisms described for H_2O_2 cell death in CHO cells. Indeed, Sánchez-Góngora *et al.* (24) showed that millimolar H_2O_2 caused CHO cell death through depletion of ATP and NAD, while lipid peroxidation might not be responsible. In many cell types, mitochondria have been found to be especially sensitive to oxidative damage. Moreover, depletion of NAD and/or ATP stores appears to be a requisite for oxidant-induced necrotic cell death (25). Interestingly, ATP-synthase, α-ketoglutarate dehydrogenase and aconitase seem to be the most susceptible enzymes to be inhibited by oxidative damages (26-28). A decrease in these activities can be expected to depress the mitochondrial respiratory activity, finally resulting in cell death. So, overexpression of PRDX5 in the cytosol and mitochondria might protect cells by preventing the depletion of NAD and/or ATP, due to the reduction of H_2O_2. We have also shown that overexpression of PRDX5 in the nucleus conferred some protection to H_2O_2 cytotoxicity. This protection might be due to a highest PRDX5 content in the cytosol of these cells as compared with Control clone, related to nuclear PRDX5 that had not been transported to nucleus. It is noteworthy that DNA protection conferred by PRDX5 is responsible for the observed reduced cell death, because DNA damage does not seem to be the cause of the cytotoxicity in H_2O_2-injured CHO cells (29).

PRDX5 overexpression in the cytosol and in mitochondria reduced cytotoxicity induced

by *t*BHP, with the latter being the most effective. Therefore, these results also suggest that mitochondria might be the primary target for *t*BHP in CHO cells. Indeed, similar results were obtained by Arai *et al.* (20), who showed that overexpression of the mitochondrial GPX4 conferred a better protection against *t*BHP than cytosolic GPX4. However, cytotoxicity mechanism of *t*BHP in CHO cells still remains unclear. Firstly, *t*BHP can form alkoxyl ($RO^{\cdot}$) and peroxyl radicals ($ROO^{\cdot}$) through Fenton-type chemistry, leading to lipid, protein and DNA oxidations, and subsequent cell death (30). In Chinese hamster cells, peroxidation is weakly induced by *t*BHP, probably because of the existence of powerful antioxidative systems. Proteins seem to be the main target of oxidant action (31). A second mechanism would imply *t*BHP detoxification by GPX, leading to alterations of glutathione redox balance, protein thiol/disulfide shifts, mitochondrial permeability transition (MPT) pore and finally cell death (32, 33). In agreement with this mechanism, Tamura *et al.* (34) showed that overexpression of glutathione reductase (GR) in mitochondria of CHO cells conferred much more protection against *t*BHP than overexpression of GR in the cytosol. As reviewed by Kowaltowski *et al.* (35), MPT can be caused by non specific pore opening, resulting from oxidation of thiol groups of inner membrane proteins. The nature of the membrane permeabilization leading to MPT still remains unknown but probably includes adenine nucleotide translocase (ANT), which can be oxidized on some cysteine residues (36). Thus, overexpression of PRDX5 in cytosol and in mitochondria might protect cells by maintaining the redox balance and/or protecting mitochondrial protein like ANT from oxidation, preventing the MPT and the subsequent cell death.

Overexpressing PRDX5 caused an increase in catalase activity and, when overexpressed in the cytosol, it caused an increase in glutathione peroxidase activities. These results were quite unexpected as overexpression of other antioxidants did not modify the antioxidant

content (19-21). These higher catalase and GPX activities might be explained by a PRDX5 overexpression induced oxidative stress. However, TBARS measurement results did not reveal a higher oxidative stress in cells overexpressing PRDX5. A second potential explanation for the observed higher catalase and GPX activities could involve an upregulation of the expression of these enzymes. Although this assumption should be experimentaly ascertained, we can predict that an upregulation of catalase expression by PRDX5 is unlikely, because H_2O_2 is known to up-regulate catalase expression. A third potential explanation could involve a protection for catalase and GPX conferred by PRDX5 overexpression. As a matter of fact, catalase and GPX can be inactivated by oxidative stress (22, 23).

Overexpressing PRDX5 in nucleus decreased DNA damage induced by the lowest hydrogen peroxide concentration, while overexpression in other compartments was ineffective. H_2O_2 can induce DNA damage by generating hydroxyl radicals ($OH^{\cdot}$) through Fenton's reaction in presence of copper bound to DNA (37). Nuclear PRDX5 probably reduced DNA damage by reacting with H_2O_2 in nucleus, and thereby, limits $OH^{\cdot}$ production. The two concentrations of H_2O_2 gave similar % of tail DNA, revealing a saturation of tail DNA. This probably reflects that high levels of DNA damage occur. It is likely that with lower H_2O_2 concentration, nuclear PRDX5 would confer a better protection.

Moreover, overexpression of PRDX5 in nucleus conferred a good protection against DNA damage induced by *t*BHP. Peroxyl radicals are responsible of DNA damage induced by *t*BHP and they can cause DNA strand breaks, abasic sites and oxidized bases (38). Nuclear PRDX5 might confer protection by reducing *t*BHP and by limiting nuclear *t*BHP content.

In the presence of both peroxides, mitochondrial and cytosolic overexpression of PRDX5 were ineffective in protecting against induced DNA damages. Mitochondrial PRDX5

might be ineffective because of its limited localization. Cytosolic PRDX5 might limit H_2O_2 and *t*BHP diffusion through the nucleus, but it was apparently not sufficient to reduce DNA damage.

In conclusion, the present study provides evidence that PRDX5, when highly expressed, can protect cells from oxidative damage caused by high concentrations of H_2O_2 and *t*BHP. Given that mitochondria seem to be the primary target for peroxides, mitochondrial localization of PRDX5 might be of great importance for cell survival during oxidative stress. Increased catalase and glutathione peroxidase activities observed in CHO cells overexpressing PRDX5 might be due to a protective effect conferred by PRDX5. These higher activities could also be implicated in the cytoprotective effects observed in presence of peroxides. The absence of downregulation of catalase and glutathione peroxidase indicates that there is no balance between PRDX5 and the different antioxidant enzymes that have the same localization and activity. This absence of balance could be explained by the fact that PRDX5 might have other functions than the antioxidative one. Indeed, cytosolic PRDX5 has been implicated in signal transduction in mammalian cells, by controlling H_2O_2 concentration (6). When localized in the nucleus, PRDX5 is able to decrease DNA oxidative damage. This ability might protect *in vivo* DNA of epithelial cells of the kidney tissue which is submitted to high oxidative stress and where PRDX5 was also found in the nucleus (9).

Finally, this study suggests that PRDX5 might be, when highly expressed in cells, a potent antioxidant able to reduce cytotoxicity and genotoxicity in acute oxidative stress while in more physiological conditions, PRDX5 might more likely be implicated in regulating redox-responsive process.

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Legends

Fig. 1 Organization of human PRDX5 constructs in vector pEF-BOS and structure of PRDX5 proteins. A, organization of the construct cloned into mammalian expression vector pEF-BOS. EF-1α prom.: promoter region of human EF-1α chromosomal gene (1.2 kb). G-CSF-poly-A: poly(A) adenylation signal from human Granulocyte-Colony Stimulating Factor. B, representation of the different PRDX5 proteins cloned into pEF-BOS and expressed in CHO cells. pEF-BOS-Cyto-PRDX5 (1) allowed the expression of a short PRDX5 containing a peroxisomal targeting signal (PTS 1) and localized to the cytosol, peroxisomes and the nucleus while pEF-BOS-Mito-PRDX5 (2), due to the presence of a clivable (arrows) mitochondrial targeting sequence (MTS), allowed the expression of the long form of PRDX5 in mitochondria. In the pEF-BOS-Nuc-PRDX5 (3), a sequence coding for a nuclear localization signal (NLS) was fused at the 5' end of the short form. Molecular weights of the different mature PRDX5 are indicated.

Fig. 2 Immunolocalization of PRDX5 in different transfected clones of CHO cells. Cells were fixed, permeabilized and incubated with a rabbit anti-human PRDX5 antiserum and FITC-conjugated donkey anti-rabbit IgG (A). Cells nuclei were simultaneously counterstained with DAPI (B) and cells were examined by fluorescence microscopy. Control: CHO cells transfected with the empty vector pEF-BOS; Cyto: CHO cells transfected with pEF-BOS-Cyto-PRDX5; Mito: CHO cells transfected with pEF-BOS-Mito-PRDX5; Nuc: CHO cells transfected with pEF-BOS-Nuc-PRDX5. *Bar*, 10 μm.

Fig. 3 PRDX5 content is increased in CHO cells transfected with different PRDX5 constructs in pEF-BOS. A, To assess PRDX5 expression, immunoblotting was performed for Control and PRDX5 transfected CHO clones. Mature Cyto and Mito PRDX5

appeared as proteins of 17 kDa. Nuc-PRDX5 with its nuclear localization signal appeared as a protein of 18 kDa in size but in this clone the endogenous Cyto/Mito PRDX5 form was present as a 17 kDa separated band. PRDX5 protein levels were normalized with β-actin (42 kDa). Thirty micrograms of proteins from cellular extracts were loaded into each lane for 12 % SDS-PAGE, followed by immunoblotting analysis with polyclonal rabbit anti-human PRDX5 antibody and rabbit anti-β-actin and then a peroxidase-conjugated goat anti-rabbit IgG as described in 'Experimental procedures'. B, Quantification of the expression level of PRDX5. The results were expressed as relative units with the expression of Control clone assigned a value of 100 %. 1: Control clone; 2: Cyto clone; 3: Mito clone; 4: Nuc clone.

Fig. 4 Catalase activity of cell lines overexpressing PRDX5. Cyto and Mito clones show higher catalase activity. Specific catalase units were calculated as described in 'Experimental procedures''. Activities are presented as the mean ± S.E.M. of results from three independent cultures derived from each line. Significance versus control is designated as * $p < 0.05$.

Fig. 5 Glutathione peroxidase (GPX) activities of cell lines overexpressing PRDX5. 'Cyto' clone shows higher GPX activities. Specific GPX units were calculated as described under 'Experimental procedures'. Activities are presented as the mean ± S.E.M. of results from three independent cultures derived from each clone. Significance versus control is designated as * $p < 0.05$, ** $p < 0.01$.

Fig. 6 Effects of hydrogen peroxide (A) and *t*BHP (B) on cytotoxicity of PRDX5-overexpressing cells. Control clone (closed squares), Cyto clone (open squares), Mito clone (open triangles) and Nuc clone (cross) were exposed to the indicated concentration of peroxides for one hour. Cell death was determined by the release of LDH. The total LDH in

cells was determined after lysis of cells with 2 % Triton X-100. Values are mean ± S.E.M. of results from three replicates in each case. Significance versus control is designated as * $p <$ 0,01 and ** $p < 0.001$.

Fig. 7 Effects of hydrogen peroxide (A) and *t*BHP (B) on DNA damage of PRDX5-overexpressing cells. Control clone (closed squares), Cyto clone (open squares), Mito clone (open triangles) and Nuc clone (cross) were exposed to the indicated concentration of peroxides for 1 h (except for Mito clone that were only submitted to the lowest concentration of peroxides). DNA damages were determined by the comet assay, allowing detection of abasic sites and DNA strand breaks. Significance versus control is designated as ** $p < 0.001$.